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(71) Applicant: **NIPPON SUISAN KAISHA, LTD.**
6-2, Ohtemachi 2-chome, Chiyoda-ku
Tokyo(JP)

Applicant: **NISSUI PHARMACEUTICAL CO., LTD.**
11-1, Sugamo 2-chome
Toshima-ku, Tokyo(JP)

(72) Inventor: **Yamashita, Shinya**
Mezon Fuchu 301, 1-36-5, Miya-machi
Fuchu-shi, Toyko(JP)

Inventor: **Hata, Junichiro**
Jyunesu Takada A102, 8-24, Raiden-cho
Koga-shi, Ibaragi-ken(JP)
Inventor: **Kabeno, Shouko**
3-16-305, 3-chome, Kihei-cho
Kodaira-shi, Tokyo(JP)
Inventor: **Matsumoto, Ken**
2120 Shimoheimi, Souwa-machi
Sashima-gun, Ibaragi-ken(JP)
Inventor: **Yagihashi, Satoru**
Nissui Seiyaku Ryo, 474-1, Shimoheimi
Souwa-machi, Sashima-gun, Ibaragi-ken(JP)
Inventor: **Kato, Hideo**
5-16-7-202, Yutaka-cho
Kasukabe-shi, Saitama-ken(JP)

(74) Representative: **Wächtershäuser, Günter, Dr.**
Tal 29
W-8000 München 2(DE)

(54) **Method of measuring antimicrosormal antibody and process for the production of human thyroid peroxidase used for the measurement.**

(57) There are given disclosures on a method of measuring an antimicrosormal antibody, a gene engineering process for preparing human thyroid peroxidase to be used for the measurement as well as an expression of the human thyroid peroxidase. The antimicrosormal antibody is measured by checking and investigating a reactivity of the human thyroid peroxidase produced by gene engineering techniques with a human serum. A natural membrane bound type human thyroid peroxidase or a secretion type human thyroid peroxidase is produced. The natural membrane bound type human peroxidase is produced on cell membrane of an cultivated animal cell or Escherichia coli transformed with an expression vector having therein recombinant DNAs encoding the human thyroid gene and dihydrofolate reductase gene. The secretion type human thyroid peroxidase is produced in a supernatant of cultivated medium of animal cell transformed with an expression vector having therein recombinant DNAs encoding the human thyroid gene but with no membrane region thereof and dihydrofolate reductase gene.

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DNA polymerase to make each end in blunt state.

The ligation of the both DNA fragments can be carried out with use of T4 ligase. Escherichia coli (JM109 strain) was cultivated with use of reaction solution to cause a transformation thereof, screened to obtain ampicillin resistance colony, and then recovered a plasmid (pSV2-hTP01) from the transformant. The plasmid is that dhfr (dihydrofolate reductase) gene in the plasmid (pSV2-dhfr) was recombined to hTPO gene, so that the dhfr gene usable as a drug resistance marker should be inserted therein to make complete the plasmid as that for expressing hTPO.

Therefore, the plasmid (pSV2-dhfr) was digested by restriction enzymes (PvuII and BglII) to obtain a DNA fragment (about 1100bp) with a SV40 promoter region and the dhfr gene. While, said re-constructed plasmid (pSV2-hTP01) was cleaved with use of a restriction enzyme (EcoRI).

After pre-treatment in accordance with a manner known per se , both of said DNA fragments were ligated with use of T4 ligase. Escherichia coli (JM109 strain) was transformed with use of the solution for ligation reaction, screened to obtain an ampicillin resistance colony, and recovered a plasmid (pSV2-hTPO-dhfr) from the transformant.

A desired hTPO can be produced in stable and large amount, when the plasmid (pSV2-hTPO-dhfr) is inserted in a CHO cell (DXB11 strain) which is one of eukaryotic cells and widely employed as a host for production of substances with use of gene recombinant engineering techniques, and the resulting transformed cells were cultivated in a manner known per se .

In the following, another case of that an expression plasmid is inserted into Escherichia coli to produce hTPO shall be explained. In this case, also, it is started from the hTPO gene inserted plasmid (phTPO-1) disclosed by S. KIMURA et al , as in aforesaid case, the plasmid is digested and then re-constructed to prepare an expression plasmid. Namely, the plasmid (PhTPO-1) is digested with use of restriction enzymes (EcoRI and XhoI) to obtain a DNA fragment (about 2900bp) with a translational region for hTPO gene and 3′-site untranslational region. While, a plas mid (pKK223-3) which is known as an expression vector in Escherichia coli is digested with use of a restriction enzyme (EcoRI) to obtain a DNA fragment.

Prior to a ligation operation for the fragments, following DNA linker (73bp for each strand) is prepared with use of a DNA synthesizer, so as to give a translation initiation codon of ATG which is necessary for an expression of DNA encoding a mature hTPO.

EcoRI

MetArgAlaLeuAlaValLeuSerValThrLeuValMetAlaCysThr

5′ -AATTCATGAGAGCGCTCGCTGTGCTGTCTGTCACGCTGGTTATGGCCTGCACA

3′ - GTACTCTCGCGAGCGACACGACAGACAGTGCGACCAATACCGGACGTGT

XhoI

GluAlaPhePheProPhelle

GAAGCCTTCTTCCCCTTCATC

CTTCGGAAGAAGGGGAAGTAGAGCT

In the presence of a phosphorylation reaction of the synthetic linker, the fragments as aforesaid are ligated with use of T4 ligase. Further, Escherichia coli (JM109 strain) was transformed with use of the ligating reaction solution, as in aforesaid case and an ampicillin resistance colony was obtained through a screening to recover a plasmid (pNKT-1) from the transformant, as in the manner given in the preceding case.

The desired hTPO can be produced by cultivating said transformed Escherichia coli , or by inserting said recombinant plasmid (pNKT-1) into Escherichia coli (JM109 strain) and cultivating the same.

For expressing in Escherichia coli a polypeptide including a portion for determining the antigen capable to react with autoan tibodies of a patient, a part of the hTPO gene and not the entire there of may also be inserted into a plasmid to insert the same, in turn, into Escherichia coli . In this case, a plasmid (pKK233-2) may be employed as the expression vector and this plasmid vector shall be digested by a restriction enzyme (EcoRI) to make the same into a DNA fragment.

Further, if the plasmid (phTPO-1) with hTPO gene, disclosed by S. KIMURA et al in said literature, is digested with use of restriction enzymes (XhoI and AccI), a DNA fragment (about 2450bp) with a part of an open reading frame for the hTPO gene can be obtained, and a DNA fragment (about 970bp) with also a part of the open reading frame for the hTPO gene can be obtained, when the plasmid shall be digested with use of restriction enzymes of SmaI and AccI. Please note that these DNA fragments are obtained through a treatment by the restriction enzyme of AccI, so that those contain no membrane bound region of the hTPO gene.

These DNA fragment can be pre-treated in a manner known per se and ligated with the DNA fragment of said expression vector plasmid to re-construct into recombinant plasmids (pKT-XA and pKT-SA).

When the recombinant plasmid is inserted in Escherichia coli (JM109 strain) and the resulting

with the hTPO expression vector, and subjecting a supernatalnt in the cultivation medium to an affinity column treatment to adsorb the hTPO and then cause an elution thereof.

Fig. 1 is an illustration showing a manner to construct an expression vector which is suitable to express hTPO protein in an animal cell;

Fig. 2 is patterns showing results of that a molecular weight of hTPO expressed in transformed CHO cells was measured in accordance with western blotting method, in addition to molecular weight of controls, namely hTPO isolated from human thyroid tissue and product of non-transformed CHO cells;

Fig. 3 is an illustration showing a manner to construct an expression vector which is suitable to express in Escherichia coli ;

Fig. 4 is an illustration showing manners to construct 2 expression vectors, in each of which a part of the hTPO gene is inserted;

Fig. 5 is a graph showing a result of computical analysis for amino acids encoding hTPO gene, searched from the view point of hydrophilicity - hydrophobicity of the amino acids;

Fig. 6 is an illustration showing a manner to construct an expression vector to express a secretion type hTPO;

Fig. 7 is patterns showing results of measurements, in accordance with a coomassy dyeing method, on molecular weight of a secretion type hTPO, a natural membrane bound type hTPO, a membrane bound type hTPO digested by trypcin as well as various molecular weight markers;

Fig. 8 is patterns showing results of measurements, in accordance with the western blotting, on molecular weight of the secretion type hTPO, natural membrane bound type hTPO, and membrane bound type hTPO digested by trypcin as well as various molecular weight markers; and

Fig. 9 is a graph showing results of search on reactivity of the secretion type hTPO and natural membrane bound type hTPO with serum samples of normal persons and serum samples of patients with autoimmune thyroid diseases.

The invention will now be further explained by Examples for preparing an hTPO expression vector, for inserting the vector into an animal cell or Escherichia coli , Example for measuring antimicrosormal antibodies, which uses the expressed hTPO, Test Examples and the like. Please note that Examples 1 - 6 as well as Test Examples 1 - 3 relate to a membrane bound type hTPO, but Examples 7 and 8 as well as Test Examples 4 and 5 to a secretion type hTPO.

Example 1

(Preparation of expression vector)

This Example shall be explained with reference to Fig. 1.

An hTPO gene cloned by S. KIMURA et al has been inserted at EcoRI recognition site of a plasmid (PhTPO-1) with 5800bp.

To a solution of this plasmid (10μ g) in a mixture (50μ l) of 10mM Toris chloride buffer (pH 7.5), 7mM $MgCl_2$ and 60mM NaCl, a restriction enzyme (EcoRI, 10 units) was added to digest the plasmid at 37° C for 2 hours. A DNA fragment (about 2μ g) with the hTPO gene and of about 3100bp was obtained from the reaction solution by a DNA separation method of agarose gel electrophoresis.

While, to a solution of a plasmid (pSV2-dhfr, 10μ g) known as one of vectors expressing a products in animal cells, in a mixture (50μ 1) of 10mM Toris chloride buffer (pH 7.5), 7mM $MgCl_2$ and 60mM NaCl, restriction enzymes (HindIII and BglII, each 10 units) were added to digest the plasmid at 37° C for 2 hours. A DNA fragment (about 3μ g) with SV40 promoter region, terminator region and replication origin in Escherichia coli and of about 4000bp was obtained from the reaction solution by the DNA separation method of agarose gel electrophoresis, as in the above.

To each of both DNA fragments (1μ g, respectively), DNA polymerase I (for large fragment) and a mixture of 6.6mM $MgCl_2$, 6.6mM Toris chloride buffer (pH 7.5), 50mM NaCl, 6.6mM mercaptoethanol and 500μ M dNTP were added to react at 23° C for 60 minutes. After the reaction, an extraction was carried out with phenol to inactivate the enzyme, and purify the DNA with use of the ethanol precipitation method to obtain each DNA fragment (about 0.5μ g) with blunt end.

To a mixture (50μ l) of 70mM Toris chloride buffer (pH 7.5), 10mM $MgCl_2$, 10mM DTT and 1mM ATP, each of said DNA fragments (0.5 μ g, respectively) was added to dissolve the same, and T4 ligase (5 units) was added therein to react at 16° C for 18 hours. With use of the reaction solution, Escherichia coli (JM109 strain) was transformed and an ampicillin resistance colony was obtained to recover a plasmid from the

prepare 400μ g/ml solution.

The resulting vector solution and said cell suspension were mixed in equiamount and subjected to an electroporation. The electroporation was carried out with use of a cell fusing device (Type SSH-1) marketed by Shimazu Manufacturing Co., Ltd. and by setting pulse intensity of 3KV/cm, pulse width 50 μ sec, pulse interval 1.0sec and number of pulse 2 times. The mixture (40μ l) was added in an insertion chamber (type C-12) and was left to stand, after the application of the pulse, under room temperature for about 10 minutes and then transferred, in equiamount, to 10 petri dishes (100mm in diameter) which accommodate the 5% D-FCS added α (+) MEM medium. After cultivating for about 48 hours, the medium was changed to α (-) MEM medium (marketed by GIBCO Co. No. 410-2000), in which 5% D-FCS was added, and the cultivation was continued, while changing the medium with a fresh one with an interval of 3 days. After 2 weeks from the initiation of cultivation, a colony of transformed cells was separated with use of a penicillin cup and recovered through trypcin treatment. The colony was subcultured with use of a multidish with 12 cell portions and then subjected to a screening, as stated below.

b) Screening of hTPO expression cell

The hTPO is a membrane enzyme of human thyroid follicular cells, so that a detection of hTPO expressed on CHO cells was carried out with use of a membrane immuno fluorescent assay (herein after referred to as --MIFA--).

In the first place, the transformed cells cultivated with the multidish were washed with PBS, recovered by a rubber policeman, suspended in PBS (100μ l), transferred to one of cell portions in a titer plate with 96 cell portions, centrifuged (1000rpm for 5 minutes) to remove supernatant therein, and suspended again in PBS (100μ l). After repeating 3 times of the centrifugal and suspending operations, a solution (100μ l) of rabbit anti-hTPO serum diluted 20 volumes with PBS was added and the resulting solution was left to stand for 1 hour at room temperature. After centrifugal washing 3 times with PBS, a solution (50μ l) of FITC labeled anti-rabbit IgG antibody (marketed by Medical and Biological Laboratory Co., Ltd.) diluted 20 volumes with PBS was added and the resulting solution was left to stand for 1 hour at room temperature. The centrifugal washing with PBS was repeated 3 times, and then the cells were suspended in 50μ l of PBS containing 50% glycerol, and a cell accommodating the cell suspension was mounted on a non-fluorescent slide glass to radiate an exciting lightbeam with use of a fluorescent microscope (marketed by Nikon Co., Ltd.).

A fluorescent cell is the desired one transformed and with an ability for producing hTPO.

Test Example 1

(Measurement of Molecular weight for produced hTPO)

The hTPO expressed cells confluently propagated in the petri dish [Item (b) in Example 2] were washed with PBS to add an icecooled CSS solution [10ml, containing 10mM Toris chloride buffer (pH 7.5), 150mM NaCl, 0.5% Toriton X-100 and 0.2mM phenylmethylsulphonylfluoride], left to stand for 15 minutes, collected with a rubber policeman, and centrifuged (1000rpm for 10 minutes) to obtain a supernatant as a cell solution.

To the cell solution (30μ l), a sample buffer [15 μ l, containing 60mg/ml SDS, 150mM Toris chloride buffer (pH 6.8) 20% glycerol and 0. 01% BPB] was added, subjected to an electrophoresis, and transcriped to a nitrocellurose membrane. The membrane was dipped in a blocking solution ("Blockace" marketed by Snow Brand Milk Products Co., Ltd.) for one overnight. The membrane was then dipped in a rabbit anti-hTPO serum diluted to 500 fold volumes with T-PBS (0.05% Tween added PBS) and mildly shaked. During the shaking, the solution of T-PBS was changed 3 times with fresh one with an interval of 15 minutes. The membrane was transferred to a solution of T-PBS containing I^{125}-labeled anti-rabbit IgG antibody (5μ Ci) to further shake mildly for 1 hour.

The resulting membrane was dried by air and an X-ray film was sensitized by the membrane at -70 °C to detect a band identifying hTPO.

As controls, CHO cells not inserted the pSV2-hTPO-dhfr vector as well as a natural type hTPO isolated from a human thyroid tissue in accordance with the method as disclosed in J. Clin. Endocrinol. Metab., Vol. 63, page 570 (1986) were employed.

Table

Samples		Test	Control
1	C	+ + +	+
2	G	+ +	+
3	G	+ + +	-
4	G	+ +	-
5	G	±	-
6	G	+ + +	-
7	G	+ + +	-
8	G	+ +	±
9	C	±	±
10	C	+	+
11	C	+	-
12	C	+	+
13	C	+ +	-
14	C	+ + +	±
15	H	+ + +	+
16	H	+ +	-
17	G	+ +	±
18	G	+ + +	-
19	G	+ +	-
20	G	+	-
21	C	+ + +	-
22	C	+ + +	-
23	H	+ + +	-
24	C	+ +	-
25	H	+ +	±
26	G	+ + +	±
27	H	+ + +	±
28	C	+ +	±

In the Table,

G : Basedow's disease,
C : Chronic thyroiditis,
H : Hyperthyroidism,
- : Almost no fluorescence,
± : Recognizable fluorescence,
+ : Apparent fluorescence, and
+ + : Strong fluorescence, and
+ + + : Very strong fluorescence.

Example 3

[Acquisition of cell strain with high hTPO expression ability, from methotrexate (MTX) resistance cell strains]

The hTPO expression cells (1 x 10^5 cells) obtained by Example 2 was suspended in a medium (8ml) and transferred to a petri dish (10cm in diameter). The medium was prepared by adding 0.05μ M MTX in 5% FCS added α (-) MEM medium. The medium was changed to a fresh one with an interval of 3 days to

yeast extract and 5g/l NaCl), cultivated at 37° C to add isopropylthiogalactopyranoside (IPTG) in concentration of 50μ g/ml at a middle of logarithmic growth phase thereof and to continue the cultivation at 37° C and then centrifuged to collect a solid. The solid was suspended in a sample buffer of laemli, subjected to SDS polyacryloamide gel electrophoresis, subjected to western blotting in accordance with the manner as described in "Proc. Natl. Acad. Sci. USA" Vol. 76, page 4350 (1979), and subjected to percxidase dyeing in accordance with the manner as described by TABE in the literature "Saibou Kougaku" (translated as --Cell Engineerings--), Vol. 2, page 1061 (1983) to detect a band at position of molecular weight of 107K daltons. This means that the Escherichia coli with plasmid (pNKT-1) expresses hTPO.

Example 6

(Expression of polypeptide constituting a part of hTPO)

An experiment was carried out for the purpose of expressing in Escherichia coli a polypeptide containing an antigen determination site reacting with an autoantibody of patient, and for producing not the entire DNA for hTPO but a part thereof.

This Example shall be explained with reference to Fig. 4. In the first place, a hTPO gene inserted plasmid (phTPO-1, 5 μ g) disclosed by S. KIMURA et al was dissolved in a mixture (50 μ l) of 10mm Toris chloride buffer (pH 7.5), 7mM $MgCl_5$ and 100mm NaCl, and then restriction enzymes (XhoI and AccI, each 10 units) were added to the solution to digest the plasmid at 37° C for 2 hours. From the reaction solution, a DNA fragment (about 1μ g, 2446bp) with a part of hTPO translational region was obtained by a DNA separation method of agarose gel electrophoresis.

Similar to the above using the plasmid (phTPO-1, 5μ g) but using restriction enzymes of smaI and AcciI (each 10 units), a DNA fragment (about 1μ g, 972bp) with a part of hTPO translational region was obtained from a reaction solution.

z Each of the both DNA fragments was dissolved in a mixture (50μ l) of 6.6mM $MgCl_5$, 6,6mM Toris chloride buffer (pH 7.5), 50mM NaCl, 6.6mM mercaptoethanol and 500μ M dNTP, and DNA polymerase I (for large fragment, 1 unit) was added to the solution to react at 23° C for 60 minutes. A phenol extraction was carried out on the reaction solution to inactivate the enzyme and the resulting extract was purified in accordance with ethanol precipitation method to obtain a DNA fragment (each 0.5μ g) with a blunt end.

While, to a solution of a plasmid (pKK223-2, 1μ g, marketed by Pharmacia Co.) in a mixture (50μ l) of 10mm Toris chloride buffer (pH 7.5), 7mM $MgCl_2$ and 100mm NaCl, a restriction enzyme (NcoI, 10 units) was added to digest the plasmid at 37° C for 2 hours. After inactivation of the enzyme by phenol extraction, ethanol precipitation method was carried out to purify and obtain a DNA fragment (pKK223-2, about 0.5μ g). The DNA fragment (about 1μ g) was dissolved in a mixture (50μ l) of 6.6mM $MgCl_2$, 6,6mM Toris chloride buffer (pH 7.5), 50mM NaCl, 6.6mM mercaptoethanol and 500μ M dNTP, and DNA polymerase I (1 unit) was added to the solution. A phenol extraction was carried out on the reaction solution to inactivate the enzyme and the resulting extract was purified in accordance with ethanol precipitation method to obtain a DNA fragment (about 0.5μ g) with a blunt end (this DNA fragment shall be referred to as "vector DNA fragment" for the sake of explanation).

The vector DNA is ligated with one of aforesaid 2 DNA fragments (with 2446bp and 972bp, respectively and with a blunt end) to re-construct 2 different expression vectors, but a manner of the ligation is same with each other. Namely, the vector DNA fragment and one of the DNA fragments, for instance that with 2446bp are dissolved in a mixture (50μ l) of 70mM Toris chloride buffer (pH 7.5), 10mM $MgCl_2$, 10mM DTT and 1mM ATP, and T4 ligase (5 units) was added thereto to react at 16° C for 18 hours to cause a ligation of the fragments. Escherichia coli (JM109 strain) was transformed with use of the resulting reaction solution and an ampicillin resistance colony was obtained through a screening. This transformant is the desired transformed Es cherichia coli .

When a plasmid is recovered from this transformant, a desired recombinant expression plasmid (pKT-XA) can be obtained. While, if starting from the DNA fragment with 972bp, another desired plasmid (pKT-SA) can finally be obtained.

A structure of the plasmid (pKT-XA and pKT-SA) has been checked and confirmed through a digestion with use of various restriction enzymes.

Test Example 3

region for hTPO gene but with no membrane bound region was obtained through a DNA separation method of agarose gel electrophoresis.

While, to a solution of a plasmid (pSV2-dhfr, loin g) known as one of vectors expressing a product in animal cells, in a mixture (50μ l) of 10mm Toris chloride buffer (pH 7.5), 7mM $MgCl_2$ and 60mm NaCl, restriction enzymes (HindIII and BglII, each 10 units) were added to digest the plasmid at 37° C for 2 hours. A DNA fragment (about 3μ g, about 4kb) with SV40 promoter region, terminator region and replication origin in Escherichia coli was obtained from the reaction solution by the DNA separation method of agarose gel electrophoresis.

To each of this DNA fragment and aforesaid hTPO gene containing DNA fragment (1μ g, respectively), DNA polymerase I (for large fragment) and a mixture (50μ l) of 6.6mM Toris chloride buffer (pH 7.5), 6.6mM $MgCl_2$, 50mM NaCl, 6.6mM mercaptoethanol and 500μ M dNTP were added to react at 23° C for 60 minutes. After the reaction, an extraction was carried out with use of phenol to inactivate the enzyme and an extract was purified through ethanol precipitation method to obtain each DNA fragment (about 0.5μ g) with blunt end.

To a mixture (50μ l) of 70mM Toris chloride buffer (pH 7.5), 10mM $MgCl_2$, 10mM DTT and 1mM ATP, each of said DNA fragments (0.5 μ g, respectively) was added to dissolve the same, and T4 ligase (5 units) was added therein to react at 16° C for 18 hours. With use of the reaction solution, Escherichia coli (JM109 strain) was transformed and an ampicillin resistance colony was obtained to recover a plasmid from the transformant (This recombinant plasmid had been named as "pSV2-hTPO-EAL".

This plasmid corresponds to that the part of dhfr (dihydrofolate reductase) in the plasmid (pSV2-dhfr) was substituted with the hTPO gene. Since the dhfr gene to be cleaved off through said treatment is useful to give a drug resistance, an operation for inserting this gene into the plasmid (pSV2-hTPO- EAL) shall be explained.

In the first place, the plasmid (pSV2-dhfr, 5μ g) was dissolved in a mixture (50μ l) of 10mM Toris chloride buffer (pH 7.5), 7mM $MgCl_2$ and 100mM NaCl, and restriction enzymes (PvuII and BglII, each 10 units) were added to digest the plasmid at 37° C for 2 hours. The resulting reaction solution was subjected to agarose gel electrophoresis to obtain a DNA fragment (about 2μ g, about 1.1kb) with SV40 promoter region and dhfr gene region. While, aforesaid recombinant plasmid (pSV2-hTPO-EAL, 1μ g) was dissolved in a mixture (50μ l) of 10mM Toris chloride buffer (pH 7.5), 7mM $MgCl_2$ and 100mM NaCl, and a restriction enzyme (EcoRI, 10 units) was added to digest the plasmid at 37° C for 2 hours, so that the plasmid (pSV2-hTPO-EAL) was cleaved at its EcoRI recognition site.

To each of both DNA fragments (1μ g, respectively), DNA polymerase 1 (for large fragment, 1 unit) and a mixture (50μ l) of 6.6mM $MgCl_2$, 6.6mM Toris chloride buffer (pH 7.5), 50mM NaCl, 6.6mM mercaptoethanol and 500μ M dNTP were added to react at 23° C for 60 minutes. After the reaction, an extraction was carried out with phenol to inactivate the enzyme, and an extract was purified through ethanol precipitation method to obtain each DNA fragment (about 0.5μ g) with blunt end.

To a mixture (50μ l) of 70mM Toris chloride buffer (pH 7.5), 1mM $MgCl_2$, 10mM DTT and 1mM ATP, each of said DNA fragments (0.5 μ g, respectively) was added to dissolve the same, and T4 ligase (5 units) was added therein to react at 16° C for 18 hours. With use of the reaction solution, Escherichia coli (JM109 strain) was transformed and an ampicillin resistance colony was obtained to recover a plasmid as a desired expression vector, from the transformant (This recombinant plasmid had been named as "pSV2-hTPO-EAL-dhfr").

Example 8

a) Cultivation of CHO-dhfr cell

Same with that as described in Reference Example.

b) Insertion of expression vector and acquisition of transformants

Same with that as described in Item a) in Example 2, accepting that the vector (pSV2-hTPO-EAL-dhfr) obtained by Example 7 was inserted in CHO cell, in lieu of the vector (pSV2-hTPO-dhfr) for Example 2.

solution (50μ l) containing hTPO in a concentration of 100ng/μ 1. To this hTPO solution, a sample buffer solution [15μ l with a composition of 60mg/ml SDS, 150mm Toris chloride buffer (pH 6.8), 20% glycerol and 0.01% BPB] was added and the resulting mixture was subjected to 8% polyacryloamide gel electrophoresis. A transcription to a nitrocellurose membrane was carried out and the membrane was dipped in a blocking solution [same with that referred in Item c) of Example 8)] for one overnight. The membrane was then dipped in a rabbit anti-hTPO serum diluted in 500 volumes with T-PBS (0.05% Tween added PBS) and mildly shaked for 1 hour (In this case, T-TBS was changed 3 times with an interval of 15 minutes). The membrane was dipped in biotinized anti-rabbit IgG solution diluted in 500 volumes to mildly shake the same for 1 hour. After washed 3 times with PBS, the membrane was reacted with ABS reagent for 30 minutes, and then washed again 3 times with PBS. The resulting membrane was dipped in a substrate solution [5mg dianisidine, 30μ l H_2O_5, and 1ml/100ml 1M Toris chloride buffer (pH 7.5)] to leave to stand for 15 minutes at room temperature to detect a band. Results are shown in Fig. 8. In the above experiments according to western blotting, an ABC kit (marketed by Vector Lab. Co.) was employed.

Results of another experiments, wherein aforesaid hTPO solution was subjected to electrophoresis, as in the above experiment, and then treated in accordance with a coomassy dyeing method, are shown in Fig. 7.

In both experiments, a natural membrane bound type hTPO isolated from human thyroid tissue in accordance with the method as disclosed in "J. Clin. Endocinol. Metab.", Vol. 63, page 570 (1986) had been employed as a control.

As apparently seen from Figs. 7 and 8, the natural type hTPO protein shows a molecular weight of 107K daltons, but the molecular weight of secretion type hTPO reduces therefrom by that for the membrane bound region to show 101K daltons.

Test Example 5

(Reactivity of membrane bound type hTPO and secretion type hTPO with serum of patient with autoimmune thyroid diseases)

A reactivity of the secretion type hTPO and natural membrane bound type hTPO with a serum sample from patients with autoimmune thyroid diseases was checked in accordance with the enzyme immuno assay similar to that as referred to in Item c) of Example 8, excepting that serum of patients with autoimmune thyroid diseases diluted in 10 volumes with PBS was employed in lieu of the rabbit anti-hTPO serum, biotinized human anti-IgG solution was employed in lieu of the biotinized rabbit anti-IgG solution, and that an absorbance was measured at 492nm to make an index showing an antigenous factor of hTPO, in lieu of the measurement wave length of 490nm. Results are shown in Fig. 9. As apparently seen from the Figure, normal serum sample shows almost no reactivity with each of the hTPOs but, on the contrary thereto, serum sample from patient with autoimmune thyroid diseases shows definite reac tivity with each of the secretion type hTPO and natural membrane bound type hTPO.

The deposition numbers on the CHO-cell-lines have been given, as follows:

a) CHO cell-line transformed with a vector plasmid to express a membrane bound type hTPO:
Deposition No. FERM 8P-3076
Deposition date: August 24, 1990

b) CHO cell-line transformed with a vector plasmid to express a secretion type hTPO:
Deposition No. FERM BP-3077
Deposition date: August 24, 1990

Claims

1. A process for the measurement of an antimicrosormal antibody by using as an antigen a human thyroid peroxidase (hTPO) and checking a reactivity of the antigen with a human serum sample, said hTPO being prepared through steps of cleaving a vector which has an insert of a cloned hTPO gene, with an restriction enzyme to prepare a DNA fragment encoding at least a part of a gene for hTPO; cleaving another vector expressing a product by an animal cell or Escherichia coli , with at least one restriction enzyme to prepare another DNA fragment with a promoter region, terminator region and replication origin; ligating said DNA fragments to reconstruct into a vector; repeating, if necessary the cleaving and ligating operations to

which comprises steps of cleaving a plasmid (pHTPO-1) having an insert of cloned human thyroid peroxidase (hTPO) gene, with a restriction enzyme (AccI) to prepare a DNA fragment of about 5.7kb; ligating the DNA fragment with a synthetic linker; cleaving the resulting DNA fragment with a restriction enzyme (EcoRI) to prepare a DNA fragment of about 2.8kb and containing a hTPO translational region; reacting the DNA fragment, in the presence of T4 DNA ligase, with a plasmid (pUC13) digested by a restriction enzyme (EcoRI); transforming Escherichia coli (JM109 strain) with use of the reaction solution to give an ampicillin resistance to a forming transformant; recovering a hTPO gene containing plasmid (phTPO-EAL) from the transformant; cleaving the plasmid with a restriction enzyme (EcoRI) to prepare a DNA fragment of about 2.8kb; cleaving another plasmid (pSV2-dhfr) with restriction enzymes (HindIII and BglII) to prepare a DNA fragment of about 4kb; end blunting on each of said DNA fragments of about 2.8kb and about 4kb, and inactivating an enzymatic activity thereof; causing a ligation reaction of the resulting DNA fragments, in the presence of T4 DNA ligase; transforming Escherichia coli (JM109 strain) with use of the reaction solution to give an ampicillin resistance to a forming transformant; recovering a hTPO gene containing plasmid (pSV2-hTPO-EAL) from the transformant; cleaving another plasmid (pSV2-dhfr) with restriction enzymes (PvuIII and BglII) to prepare a DNA fragment and cleaving said plasmid (pSV2-hTPO-EAL) with a restriction enzyme (EcoRI); end blunting on each of the resulting DNA fragments; ligating the DNA fragments in the presence of T4 DNA ligase to prepare a recombinant plasmid; inactivating an enzymatic activity; and then purifying the recombinant plasmid.

12. A process for the preparation of a plasmid vector as claimed in Claim 11, wherein said synthetic linker has a nucreotide sequence of

5′ -- AGACTAGTGAATTC -- 3′

5′ -- TGATCACTTAAG -- 3′

13. An animal cell transformed with a plasmid vector to secretly produce a human thyroid peroxidase, which plasmid comprises an insert of two promoter regions, a region of human thyroid oxidase (hTPO) with no membrane bound region thereof, at downstream of one of the promoter regions, a dihydrofolate reductase (dhfr) gene region at downstream of the other promoter region, and a terminator region between the hTPO and dhfr regions.

14. A process for acquiring a human thyroid peroxidase (hTPO), which comprises steps of cultivating in a medium an animal cell transformed by an expression plasmid vector which has an insert of two promoter regions, a region of human thyroid oxidase (hTPO) with no membrane bound region thereof, at downstream of one of the promoter regions, a dihydrofolate reductase (dhfr) gene region at downstream of the other promoter region, and a terminator region between the hTPO and dhfr regions; and subjecting a supernatant of the medium to an affinity column chromatography to cause an adsorption of hTPO in the column and subsequent elution thereof from the column.

FIG. 1

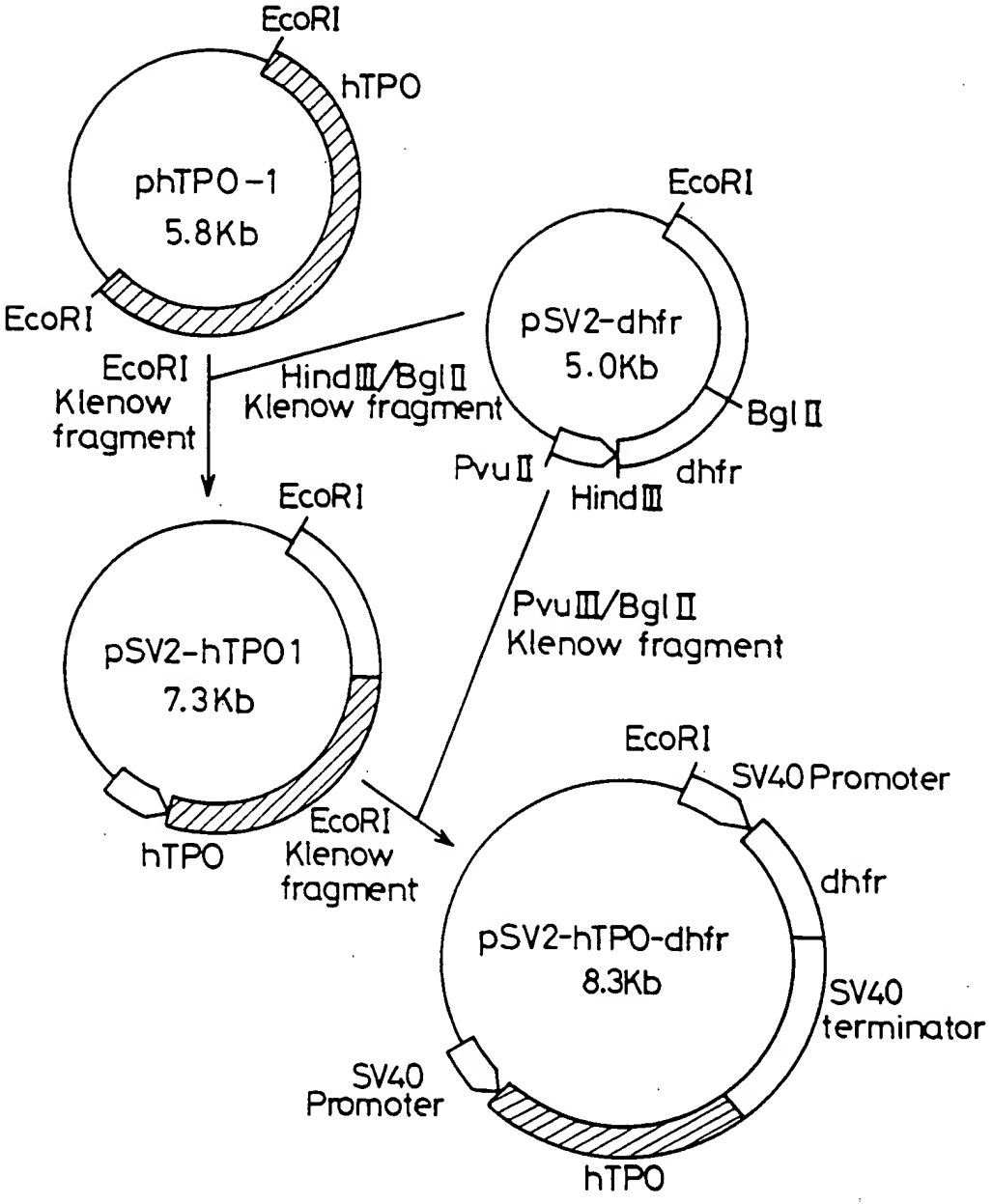
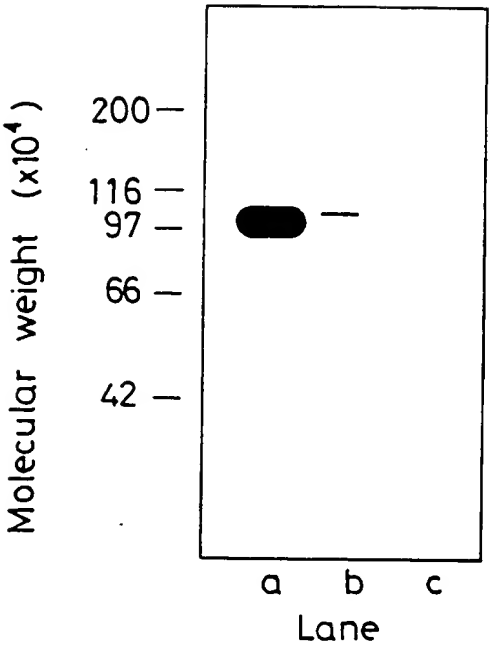

EcoRI
hTPO
phTPO-1
5.8Kb
EcoRI
EcoRI
pSV2-dhfr
5.0Kb
EcoRI
Klenow
fragment
HindIII/BglII
Klenow fragment
BglII
PvuII
dhfr
HindIII
EcoRI
pSV2-hTPO1
7.3Kb
PvuIII/BglII
Klenow fragment
EcoRI
SV40 Promoter
hTPO
EcoRI
Klenow
fragment
dhfr
pSV2-hTPO-dhfr
8.3Kb
SV40
terminator
SV40
Promoter
hTPO

FIG. 2



Lane

a : hTPO isolated from human thyroid tissue

b : Product of transformed CHO cells

c : Product of non-transformed CHO cells

FIG. 3

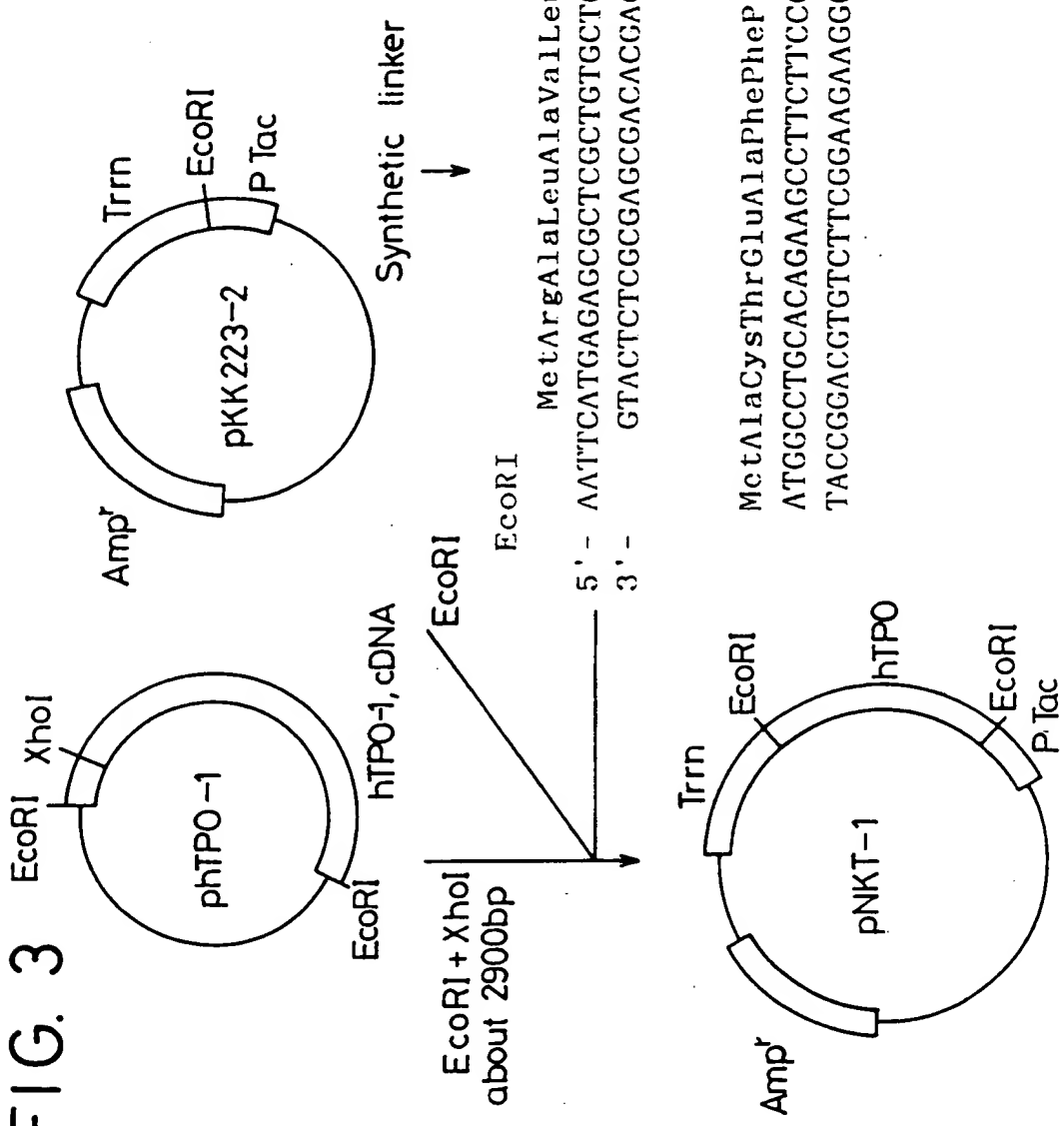

EcoRI
XhoI
phTPO-1
hTPO-1, cDNA
EcoRI
EcoRI
EcoRI + XhoI
about 2900bp
Amp^r
pKK223-2
Trrn
EcoRI
P Tac
Synthetic linker
EcoRI
Met Arg Ala Leu Ala Val Leu Ser Val Thr Leu Val
5' - AATTCATGAGAGCGCTCGCTGTGCTGTCTGTCACGCTGGTT
3' - GTACTCTCGCGAGCGACACGACAGACAGTGCGACCAA
XhoI
Met Ala Cys Thr Glu Ala Phe Pro Phe Ile
ATGGCCTGCACAGAAGCCTTCCCCTTCATC
TACCGGACGTGTCTTCGGAAGGGGAAGTAGAGCT
Trrn
EcoRI
hTPO
EcoRI
P Tac
pNKT-1
Amp^r

FIG. 4

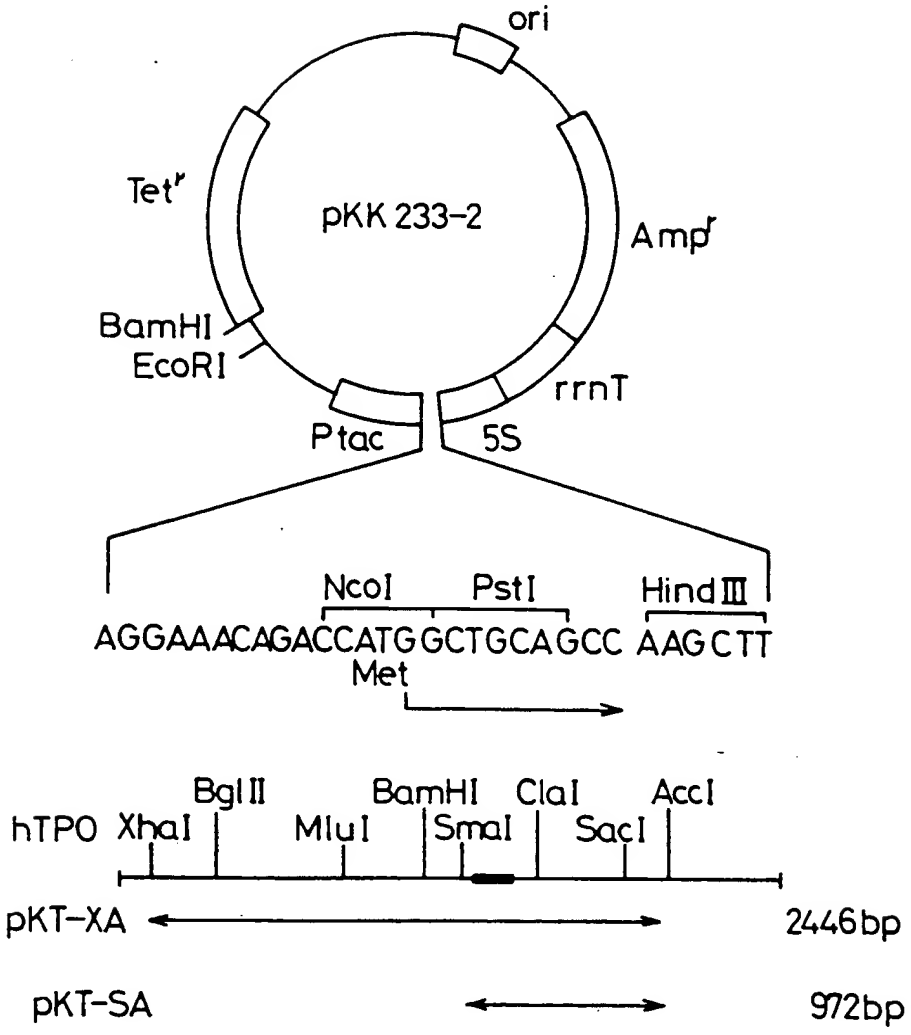
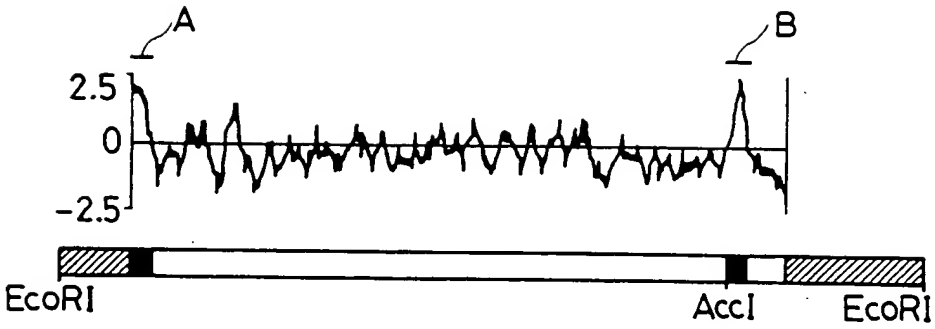

ori
Tet^r
pKK 233-2
Amp^r
BamHI
EcoRI
rrnT
Ptac
5S
NcoI
PstI
HindIII
AGGAAACAGACCATGGCTGCAGCC AAGCTT
Met
BglII
BamHI
ClaI
AccI
hTPO
XhaI
MluI
SmaI
SacI
pKT-XA
2446bp
pKT-SA
972bp

FIG. 5



A: Signal sequence
B: Membrane bound region

FIG. 6

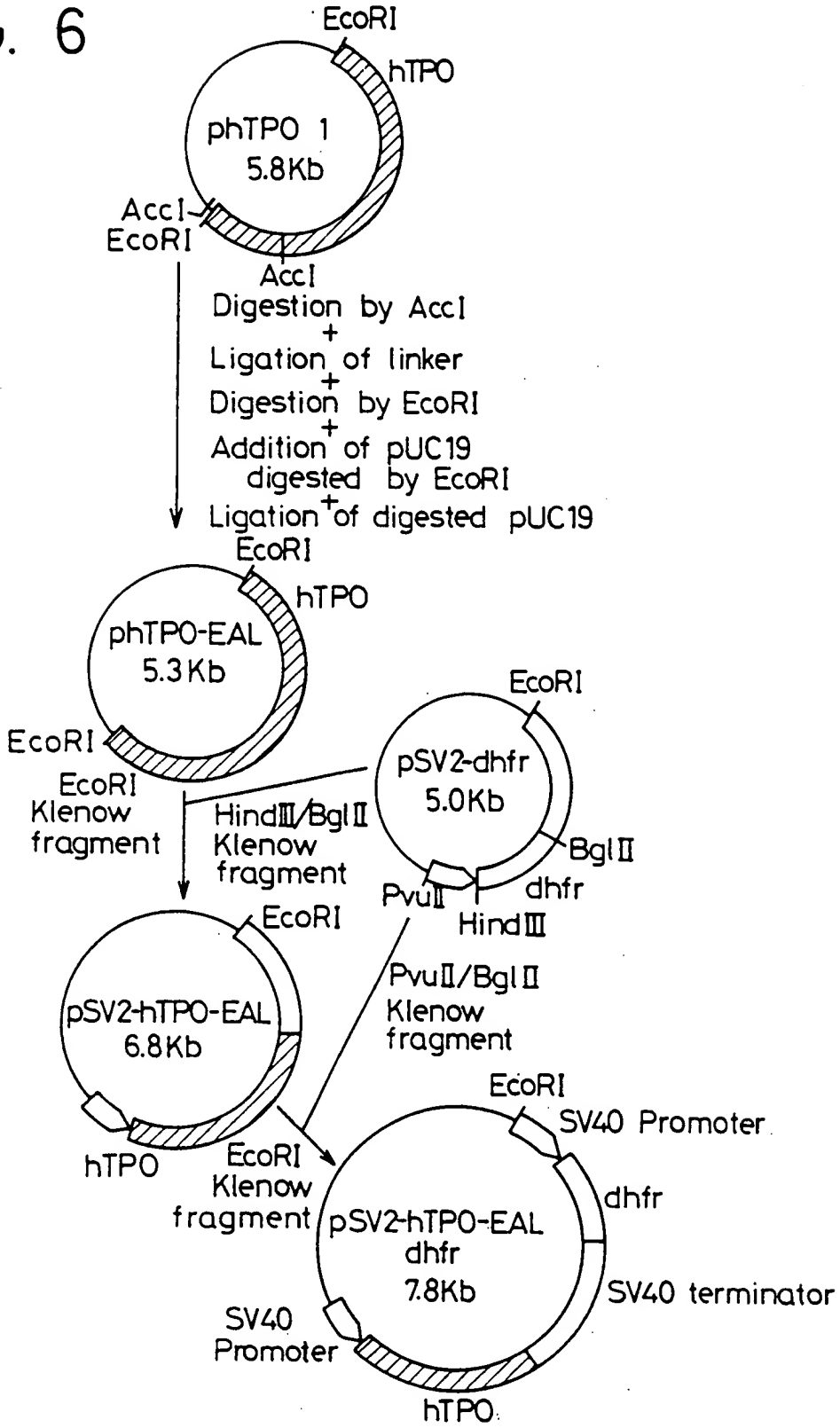
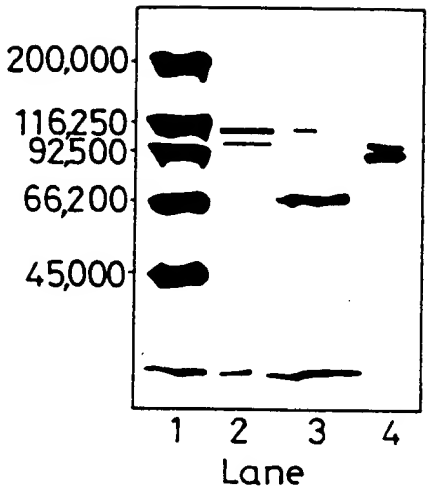
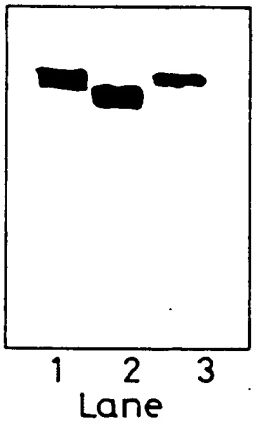

EcoRI
hTPO
phTPO 1
5.8Kb
AccI
EcoRI
AccI
Digestion by AccI
+
Ligation of linker
+
Digestion by EcoRI
+
Addition of pUC19
digested by EcoRI
+
Ligation of digested pUC19
EcoRI
hTPO
phTPO-EAL
5.3Kb
EcoRI
EcoRI
Klenow
fragment
HindIII/BglII
Klenow
fragment
EcoRI
pSV2-dhfr
5.0Kb
BglII
PvuII
dhfr
HindIII
EcoRI
pSV2-hTPO-EAL
6.8Kb
hTPO
PvuII/BglII
Klenow
fragment
EcoRI
SV40 Promoter
dhfr
EcoRI
Klenow
fragment
pSV2-hTPO-EAL
dhfr
7.8Kb
SV40 terminator
SV40
Promoter
hTPO

FIG. 7



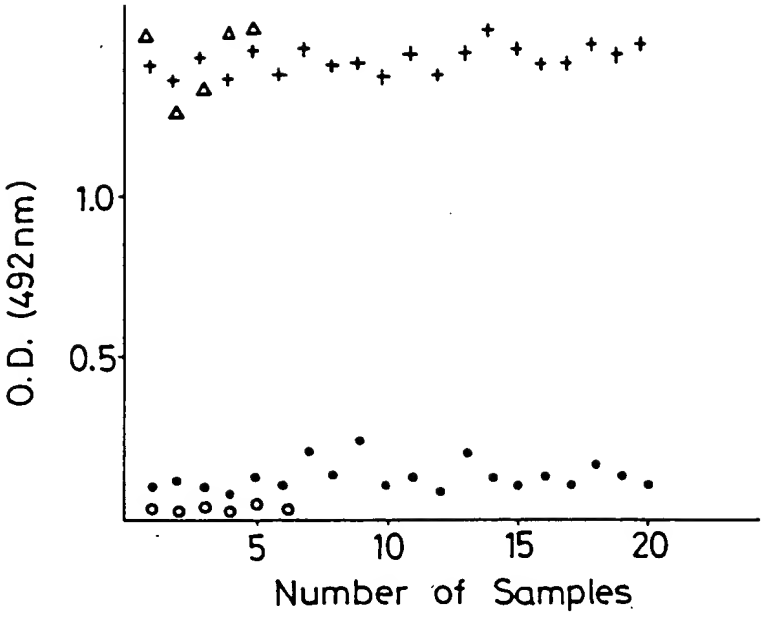
Lane 1 : Molecular weight markers
2 : Membrane bound type hTPO
3 : Secretion type hTPO
4 : Membrane bound type hTPO digested by trypsin

FIG. 8



Lane 1 : Membrane bound type hTPO
2 : Membrane bound type hTPO digested by trypsin
3 : Secretion type hTPO

FIG. 9



• : Normal serum (Membrane bound type hTPO)
+ : Patient's serum (ditto)
o : Normal serum (Secretion type hTPO)
△ : Patient's serum (ditto)

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